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The Three Subunits of the Polymerase and the Nucleoprotein of Influenza B Virus Are the

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The genes encoding the nucleoprotein, PB1, PB2, and PA proteins of the influenza virus strain B/Panamá/45/90 have been cloned under control of the T7 RNA polymerase promoter of plasmid pGEM-3. Transfection of the recombinant plasmids obtained into mammalian cells, which had been infected with a vaccinia virus encoding the T7 RNA polymerase, resulted in expression of the expected influenza B virus polypeptides. Moreover, it is shown that coexpression of the four recombinant core proteins in COS-1 cells reconstituted a functional polymerase capable of expressing a synthetic influenza B virus-like CAT RNA. By using the influenza B virus recombinant plasmids and a set of pGEM-derived plasmids encoding the homologous core proteins of the influenza A virus A/Victoria/3/75 (I. Mena *et al.* (1994). *J. Gen. Virol.* 75, 2109–2114), the capabilities of homo- and heterotypic mixtures of the four core proteins to express synthetic type A and B CAT RNAs were analyzed. Both the influenza A and B virus polymerases were active in expressing, albeit with reduced efficiencies, the heterotypic model CAT RNAs. However, none of all possible heterotypic mixtures of the core proteins reconstituted a functional polymerase. In order to fully characterize the recombinant plasmids obtained, the nucleotide sequences of the cloned genes were determined and compared to sequences of other type B virus isolates. The results obtained from these latter analyses are discussed in terms of the conservation and evolution of the influenza B virus core genes.

INTRODUCTION

Influenza viruses are classified into three different types (A, B, and C) based on the lack of serological cross-reactivity between their major structural components (the Nucleoprotein (NP) and Matrix proteins). Both influenza A and B viruses are important human pathogens and contain a genome which consists of eight single-stranded negative-sense RNA segments (Lamb, 1989; Lamb and Krug, 1996). Type A and type B viruses share structural and biochemical properties (Lamb, 1989), yet they show differences in their coding strategies (Lamb and Horvath, 1991), evolutionary patterns (Smith and Palese, 1989), and host ranges (Webster *et al.,* 1992). Moreover, natural stable reassortants between A and B viruses have never been observed (Mikheeva and Ghendon, 1982; Kaverin *et al.,* 1983).

Much of our knowledge on the mechanisms by which the influenza virus genome is transcribed and replicated derives from studies carried out on type A viruses. (Krug *et al.,* 1989; Lamb and Krug, 1996). It has been established that the functional templates for synthesis of influenza A virus RNAs are ribonucleoprotein (RNP) complexes. These RNP complexes consist of the viral genome associated with four viral-encoded proteins, the NP, which encapsidates the viral RNA (Pons *et al.,* 1969), and the three subunits (PB1, PB2, and PA) (P proteins) of the polymerase complex (Braam *et al.,* 1983; Detjen *et al.,* 1987). Recently, two major developments have allowed the characterization of the cis-signals and trans-acting factors involved in the virus-specific RNA transcription processes. On one hand, it has been possible to assemble functional influenza A virus RNP complexes *in vitro* from synthetic RNA templates and from purified P proteins and NP (Parvin *et al.,* 1989; Martin *et al.,* 1992; Seong and Brownlee, 1992). On the other hand, functional influenza A virus RNPs have been reconstituted in mammalian cells by expressing the four core proteins (PB1, PB2, PA, and NP) from cloned cDNAs (Huang *et al.,* 1990; Kimura *et al.,* 1992; de la Luna *et al.,* 1993; Zobel *et al.,* 1993; Biswas and Nayak, 1994; Mena *et al.,* 1994; Pleschka *et al.,* 1996).

Much less is known about the mechanism by which the influenza B virus genome is expressed. It is known that RNP complexes isolated from influenza B virions display transcriptase activity (Mowshowitz and Deval,

[1] The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the following accession numbers: AF005736 (PB1 gene), AF005737 (PB2 gene), AF005738 (PA gene), AF005739 (NP gene).

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1980) and that mRNAs obtained from infected cells are, as the influenza A virus counterparts, polyadenylated and contain extra nucleotides derived from host-cell mRNAs at their 5′ ends (Shaw and Lamb, 1984). Sequencing studies have revealed that the NP and P proteins of type A and B viruses share significant homologies, which range between ~37% for the NP and 61% for the PB1 subunit (Londo *et al.,* 1983; Kemdirim *et al.,* 1986; Akoto Amanfu *et al.,* 1987; Yamashita *et al.,* 1989). For both type A and B viruses, the sequences that serve as promoters for the viral polymerase are located at the 5′ and 3′ termini of the virus genomic RNAs (Parvin *et al.,* 1989; Lee and Seong, 1996). Although these cis-acting signals are not fully conserved across virus types (Desselberger *et al.,* 1980; Stoeckle *et al.,* 1987), it has been shown, using *in vitro* reconstituted RNPs, that polymerases from both A and B viruses are active in transcribing heterotypic model RNAs (Lee and Seong, 1996). Moreover, it has been shown that the influenza A virus polymerase can recognize the influenza B virus promoter *in vivo* since: (i) a synthetic type B CAT RNA could be expressed in influenza A-virus-infected cells (Muster *et al.,* 1991), and (ii) it has been possible to obtain, by RNP transfection, an influenza A virus in which the neuraminidase gene was flanked by the 5′ and 3′ terminal noncoding sequences of an influenza B virus RNA segment (Muster *et al.,* 1991).

The above observations suggest that the processes of virus-specific RNA synthesis are similar in type A and B viruses. However, the differences in nucleotide and amino acid sequences of the cis- and trans-acting factors involved, together with the differences between influenza A and B viruses mentioned above, indicate that there may be type-specific characteristics associated with these processes.

To get further insights into the mechanism by which the influenza B virus genome is synthesized, we describe here the cloning, sequencing, and functional expression of the four core proteins (NP, PB1, PB2, and PA) of the influenza strain B/Panamá/45/90 (PN/90).

MATERIALS AND METHODS

Viral strains, plasmids, and RNAs

The viral strain B/Panamá/45/90 was obtained from P. Pérez-Breña, who received it from J. J. Skehel. Vaccinia virus recombinant vTF7-3 (Fuerst *et al.,* 1986) was provided by B. Moss. Plasmids pGEM-PA, pGEM-PB1, pGEM-PB2, and pGEM-NP, which encode the core proteins of the influenza virus A/Victoria/3/75, have been described (de la Luna *et al.,* 1989, 1993; Mena *et al.,* 1994). Plasmids plVACAT1/S (Piccone *et al.,* 1993) and pT7NSBCAT (Barclay and Palese, 1995) were kindly provided by P. Palese and W. S. Barclay, respectively. These plasmids contain the coding sequence of the CAT gene (in negative polarity) flanked by the 5′ and 3′ nontranslated sequences of the virus RNA segment encoding either the influenza A (plVACAT1/S) or the influenza B (pT7NSBCAT) NS proteins. To obtain the influenza virus-like CAT RNAs, the plasmids were digested with *Hga*I and transcribed *in vitro* with T7 RNA polymerase (using the MEGAscript T7 kit, Ambion).

Cloning of influenza B virus genes

Virus genomic RNA was isolated from purified virions of the strain PN/90. An aliquot of virus RNA (12 μg) was reverse transcribed into cDNA in a reaction mixture (200 μl final volume) containing 100 m*M* Tris·HCl (pH 8.3), 10 m*M* $MgCl_2$, 20 m*M* KCl, 0.1 m*M* EDTA, 1 m*M* dithiothreitol, actinomycin D (10 μg/ml), the four dNTPs (0.8 m*M*) (including trace amounts of [α-^{32}P]dCTP), 200 units of human placental ribonuclease inhibitor, 100 units of avian myeloblastosis virus reverse transcriptase, and 14 μg of oligonucleotide 960 (5′ taggtacccggggAGCAGAAGC), which includes the sequence complementary to the nine conserved nucleotides found at the 3′ ends of all influenza B virus RNA segments (Stoeckle *et al.,* 1987) (for all the oligonucleotides indicated in this report, the sequences corresponding to influenza B virus genes are indicated in uppercase, and the unrelated sequences, which always include an *Ava*I restriction site, are shown in lowercase). Following incubation at 42° for 30 min, the mixture was phenol extracted and chromatographed through a Sephadex G-50 column. The excluded material was mixed with 100 ng of oligonucleotide 959 (5′ ggcccgggtctagaAGTAGAAACACGAGCATTTTT) (which includes the consensus sequence determined by comparison of the 21 nucleotides present at the 5′ terminus of the viral segments encoding the core proteins of the strain B/Ann Arbor/1/66) (DeBorde *et al.,* 1988), ethanol precipitated, and resuspended in 33 μl of TE containing 5 μg of RNAse A. After incubation at 37° for 10 min, the sample was completed to 100 μl with final concentrations of 10 m*M* Tris·HCl (pH 8.3), 50 m*M* KCl, 10 m*M* $MgCl_2$, and the four dNTPs (200 μ*M*) (including trace amount of [α-^{32}P]-dCTP). The mixture was incubated for 3 min at 95°, transferred to a waterbath at 85°, and supplemented with 5 units of Taq polymerase. The waterbath was left to cool down at room temperature for 15 min (so that the temperature dropped 30°). The mixture was then phenol extracted, and the ds cDNA was purified through a Sepharose CL-4B column. The ds cDNA was then digested with *Ava*I and cloned into pGEM-3 vector previously digested with *Ava*I and treated with alkaline phosphatase. Upon transformation of *Escherichia coli* cells, the ampicillin-resistant colonies were screened with ^{32}P-labeled oligonucleotides (designed according to the gene sequences determined for strain B/Ann Arbor/1/66) specific for the four influenza B virus core genes. For cloning the

NP and PB1 genes the virus genomic RNA was reversed transcribed into ss cDNA as described above except that synthesis was carried out in two independent reactions that contained either primer 1bNP (5′ taggtacccggggCAAAATGTCCAACATGGATAT) or primer 1bPB1 (5′ taggtacccggGGAGCCTTTAAGATGAATAT). The ss cDNA obtained was then amplified by PCR (20 cycles, each consisting of 2′/94°, 1′/45°, and 3′/72°) using Vent DNA polymerase (New England Biolabs) following the instructions recommended by the manufacturer. The primers included in the two PCR reactions were 1bNP and 3bclNP (5′ ggcccgggtctagaTTAATAATCGAGGTCATC), and 1bPB1 and 3bclPB1 (5′ ggcccgggtctagaGATGACCAATAACCCCAT), respectively. The amplified products were purified from LMP-agarose gels, digested with *Ava*I, and cloned into pGEM-3 vector as described above.

Transfections and CAT assays

The assays were carried out as previously described (Mena *et al.,* 1994, 1995). Briefly, 10^6 COS-1 cells were infected with vTF7-3 (multiplicity of infection 5), and transfected, by using Lipofectin, with the indicated pGEM-derived plasmids. Six hours later, the medium was removed, and cells were transfected again with 0.5 μg of the indicated model CAT RNA. At 24 hr p.i., cell extracts were prepared and tested for CAT expression using [^{14}C]-chloramphenicol and chromatography on thin-layer chromatography plates.

Sequencing

The sequences of the cDNAs were determined using the dideoxy method with specific synthetic oligonucleotide primers and the T7 sequencing kit (Pharmacia-Biotech). Sequencing reactions were carried out on both strands of the cDNAs so that 92, 97, 92, and 90% of the PA, PB1, PB2, and NP gene sequences, respectively, were determined on both strands.

Computer analyses

For constructing the evolutionary trees, the differences in nucleotides between pairs of strains were calculated and these figures were used to determine the relationships among isolates by using the neighbor-joining method. These calculations and the trees were made with the aid of the MEGA (Molecular Evolutionary Genetics Analysis) program (version 1.02) developed by S. Kumar, K. Tamura, and M. Nei (the Pennsylvania State University, University Park, PA 16802). The sequences examined were from the following viral strains (abbreviations are given in parentheses): B/Lee/40 (LE/40), B/Ann Arbor/1/66 (AA/66), B/Singapore/222/79 (SN/79), B/Ann Arbor/1/86 (AA/86), B/Yamagata/16/88 (YM/88), B/Texas/37/88 (TX/88).

RESULTS AND DISCUSSION

Cloning of the influenza B virus core genes

Virus genomic RNA isolated from virions of the strain B/Panamá/45/90 (PN/90) was used as template for the cDNA synthesis reactions. In an attempt to obtain cDNAs of the four viral core genes (PB1, PB2, PA, and NP) from a single-tube reaction, the synthesis of the first and second strands of the ds cDNA were carried with two oligodeoxinucleotide primers, which included the consensus sequences present at the ends of the four influenza B virus RNA segments to be cloned (see Materials and Methods for details). The ds cDNA obtained was ligated into the polylinker of pGEM-3 vector and used to transform *E. coli* cells. The 2000 ampicillin-resistant colonies obtained were screened with oligonucleotide-labeled probes corresponding to the four core genes, but only colonies which hybridized to the PB2 (32 colonies) and PA probes (10 colonies) were observed. Thus, cloning of the NP and PB1 genes was accomplished by RT-PCR using gene-specific primers.

Several of the recombinant plasmids obtained were characterized by restriction analyses. Four of them (pGB-PA-4, pGB-PB1-89.1, pGB-PB2-2, and pGB-NP-7), which appear to contain full-length cDNAs (according to the cloning procedure) corresponding to the PA, PB1, PB2, and NP genes, respectively, were chosen for further analyses. In these plasmids the cDNA inserts were cloned under control of the T7 RNA polymerase promoter of pGEM-3 vector.

Functional expression of the core proteins of influenza virus B/Panamá/45/90

To determine whether the selected plasmids could drive the expression of the expected recombinant proteins, COS-1 cells were infected with the vaccinia virus recombinant vTF7-3 (which encodes the T7 RNA polymerase), transfected individually with each of the plasmids, and pulse-labeled with Tran^{35}S-label. As can be observed in Fig. 1A, each of the cell extracts from transfected cultures contained a major labeled protein band (indicated by arrowheads in the figure), which was absent in the extract from cells infected with vTF7-3 but not transfected (lane M). The electrophoretic mobilities of the recombinant polypeptides were comparable with the sizes expected for the corresponding viral proteins. However, the relative mobilities of the recombinant P polypeptides did not strictly correlate with the protein sizes predicted (87.9 kDa for PB2, 84.2 kDa for PB1, and 83 kDa for PA) from the nucleotide sequences of the corresponding cDNAs (see below). It should be mentioned that a similar situation is observed for the P proteins of influenza A virus. In this case, the PB2 (86 kDa) protein migrates, in SDS–polyacrylamide gels containing 4 *M* urea, faster

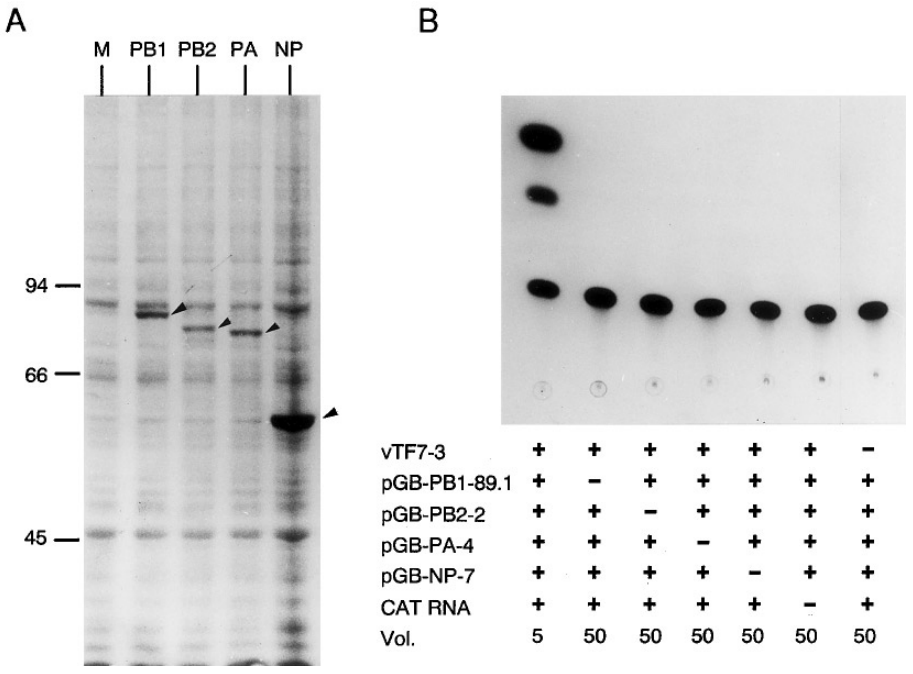

FIG. 1. Expression of influenza B virus proteins from cloned cDNAs. COS-1 cells were infected with vTF7-3 and transfected individually with 5 μg of plasmids pGB-PB1-89.1 (lane PB1), pGB-PB2-2 (lane PB2), pGB-PA-4 (lane PA), and pGB-NP-7 (lane NP), or mock transfected (lane M). At 24 hr p.i., cell cultures were incubated in methionine-free medium for 2 hr and then were pulse labeled with 14 μCi of Tran^{35}S-label for 1 hr. During all incubation steps, the medium contained 40 μg/ml of cytosine β-D-arabinofuranoside. Cell extracts were prepared, resolved in a SDS–7.5% polyacrylamide gel containing 4 *M* urea, and the labeled proteins visualized by autoradiography. Molecular weight markers, shown at left, are indicated in thousands. (B) Individual COS-1 cell cultures were infected with vTF7-3, transfected with mixtures of the pGEM-derived plasmids and a synthetic influenza B virus-like CAT RNA, as indicated in the figure. At 24 hr p.i., cell extracts were prepared (in a total volume of 100 μl) and aliquots of these extracts (5 or 50 μl as indicated in vol) were tested for CAT activity.

than the PA (82.6 kDa) and PB1 polypeptides (86.6 kDa) (Horisberger, 1980; Detjen *et al.,* 1987).

We have previously described a system in which expression of a synthetic influenza A virus-like CAT RNA is achieved in cells infected with vTF7-3, and transfected with four pGEM-derived plasmids encoding the PB1, PB2, PA, and NP genes of the viral strain A/Victoria/3/75 (Mena *et al.,* 1994, 1995). It was decided to test whether a similar artificial expression system could be established with a synthetic negative-sense influenza B virus-like CAT RNA, and the plasmids encoding the influenza B virus core proteins. As shown in Fig. 1B, significant levels of CAT expression were detected in cells coexpressing the four viral proteins and that were transfected with the type B CAT RNA. Detection of the reporter gene activity was dependent on infection with vTF7-3 and transfection of the CAT RNA (Fig. 1B). Moreover, CAT activity was not detected on omission of any of the recombinant plasmids in the transfection mixture (Fig. 1B). It was thus concluded that expression of the CAT RNA was mediated by the influenza B virus recombinant proteins. These results therefore show that the PB1, PB2, PA, and NP proteins of influenza B virus are, as previously reported for influenza A virus (Huang *et al.,* 1990; Kimura *et al.,* 1992; de la Luna *et al.,* 1993; Zobel *et al.,* 1993; Biswas and Nayak, 1994; Mena *et al.,* 1994; Pleschka *et al.,* 1996), the minimum set of viral proteins required for expression of the viral genome.

It was then determined the ratio of transfected plasmids that yielded the highest CAT activity levels. Under the optimal conditions determined (0.5 μg of plasmid pGB-PA-4, 1 μg of plasmid pGB-PB1-89.1, 0.5 μg of plasmid pGB-PB2-2, 3 μg of pGB-NP-7), incubation of an aliquot of the cell extracts (corresponding to ~20.000 COS-1 cells) with 0.1 μCi of [^{14}C]chloramphenicol (40–60 mCi/mmol) for 2 hr at 37°, yielded 4% of chloramphenicol-acetylated molecules. Similar reporter gene activities were obtained when Vero and HeLa cell cultures were used in the infection-transfection experiment (not shown).

Expression of type A and type B CAT RNAs by homo- and heterotypic polymerases

As mentioned above, the sequences at the termini of influenza virus genomic RNAs, which serve as promoters for the viral polymerase, are different in length and sequence for type A and type B viruses (Fig. 2A) (Desselberger *et al.,* 1980; Stoeckle *et al.,* 1987). However, it has been shown that the influenza A virus polymerase can

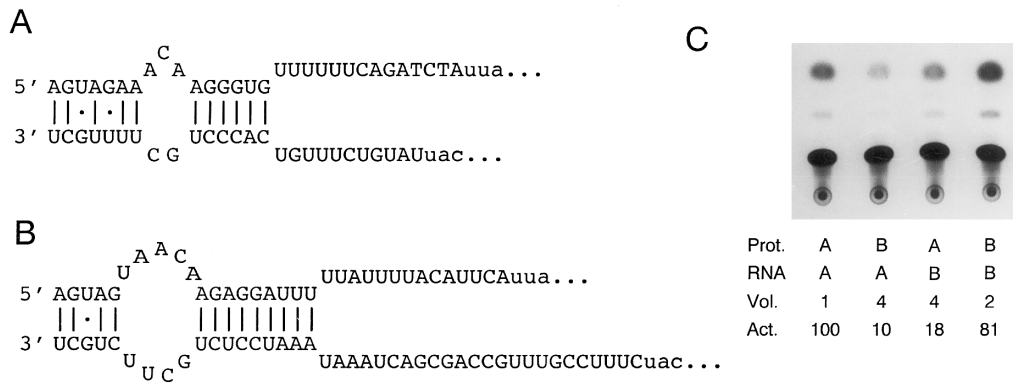

FIG. 2. Expression of heterotypic RNAs by the recombinant influenza A and B polymerases. (A, B) Nucleotide sequences and predicted panhandle structures of the type A (A) and type B (B) NS CAT RNAs. The sequences conserved in the eight viral RNA segments include the 12 and 13 nucleotides at the 3′ and 5′ ends, respectively, in type A viruses and the 9 and 10 nucleotides at the corresponding ends in type B viruses. Variation (U/C) is observed at position four of the 3′ end of influenza A virus genomic RNAs. (C) COS-1 cell cultures were infected with vTF7-3 and transfected with 4 pGEM recombinant plasmids encoding the four core proteins of either the A/Victoria/3/75 (Prot. A) or the PN/90 (Prot. B) influenza virus strains. The cultures were then transfected with the type A or type B synthetic CAT RNAs (as indicated in RNA), and cell extracts were prepared in a total volume of 100 μl. Aliquots of these extracts (1 to 4 μl, as indicated in vol) were tested for CAT expression. The CAT activity levels obtained in each sample were calculated, by taking into account the amount of cell extract used in the assay, and expressed as a percentage of the activity obtained in the culture transfected with all the influenza A virus RNP components (Act.).

recognize the influenza B virus promoter *in vivo* (Muster *et al.,* 1991) and that the polymerases from both A and B viruses are active in transcribing model type A and B RNAs *in vitro* (Lee and Seong, 1996).

The artificial system described here allowed us to use a different *in vivo* approach to study the capabilities of the two viral polymerases to express heterotypic model RNAs in the absence of other viral-encoded proteins which may modulate the expression of viral genes. For these experiments, two synthetic negative-sense CAT RNAs, which contain the nontranslated sequences of the RNA segment encoding either the influenza A or the influenza B NS proteins (Figs. 2A and 2B), were transfected into cells expressing the type A or type B recombinant core proteins. The results of a representative experiment are shown in Fig. 2C. It can be observed that cultures transfected with the homotypic components (proteins and RNA) yielded similar CAT expression activities, whereas a ~5- to 10-fold reduction in CAT expression was observed in cultures transfected with the heterotypic RNAs. It is thus clear that both type A and B polymerases can recognize, albeit with reduced efficiencies, the cis-acting signals of heterotypic model virus-like RNAs. These results agree with, and extend, those reported by Muster *et al.* (1991), which showed that the NS B CAT RNA was expressed at 1/10th the efficiency of the NS A CAT RNA in influenza A virus-infected cells.

It was of interest to determine whether heterotypic mixtures of the RNP protein components could reconstitute a functional polymerase *in vivo.* By using the eight recombinant plasmids encoding the core proteins of influenza A/Victoria/3/75 and PN/90 viruses, all possible heterotypic mixtures of the four core proteins were expressed in COS-1 cells infected with vTF7-3. The cultures were then transfected with either the type A or type B CAT RNAs and assayed for CAT expression. None of the cultures expressing heterotypic mixtures of the four core proteins yielded significant levels of CAT activity (less than 1% of the activity obtained in cells expressing all four core proteins of the same virus type) (data not shown). Although these results do not exclude the possibility that heterotypic P protein complexes can be formed in the transfected cultures, they show that the four protein components of RNPs should be derived from the same virus type in order to reconstitute a functional polymerase *in vivo.*

We consider interesting the fact that mixtures containing the three P proteins from the same virus type and the NP from the other could not support expression of either of the CAT RNAs. This result suggests that the NP is not just playing a structural role in maintaining the RNP structure but that there are type-specific interactions between the NP and the P proteins which are essential for expression of the viral genome.

Nucleotide and deduced amino acid sequences of the influenza B virus cloned genes

Sequence analyses and comparisons of the genes coding for the hemagglutinin (HA), neuraminidase, and NS proteins have revealed that the pattern of influenza B virus evolution is characterized by cocirculation of multiple evolutionary lineages for considerably long periods of time (Yamashita *et al.,* 1988; Air *et al.,* 1990; Kanegae *et al.,* 1990; Rota *et al.,* 1990, 1992). The number of sequences available for the core genes of influenza B virus

TABLE 1

Number of Nucleotide (Lower Left Half) and Amino Acid (Right Upper Panel) Differences between Pairs of NP Genes

	LE/40	AA/66	SN/79	AA/86	YM/88	TX/88	PN/90
LE/40	—	27	24	25	25	25	26
AA/66	106	—	10	16	12	11	12
SN/79	119	45	—	10	4	3	4
AA/86	130	59	42	—	12	11	12
YM/88	131	63	27	57	—	5	4
TX/88	130	62	24	60	31	—	5
PN/90	132	69	29	67	14	35	—

Note. The nucleotide sequences analyzed (1687 nt) included the polypeptide coding region (from position 60 to 1739 according to the sequence reported for the SN/79 strain). The EMBL/GenBank accession numbers for the genes analyzed were as follows: K01395 (LE/40), M20174 (AA/66), K01139 (SN/79), X14217 (AA/86), L49385 (YM/88), L49384 (TX/88), AF005739 (PN/90).

is limited, and therefore much less is known about the conservation and evolution of these genes. To gain insight into these questions, the sequences of the cloned genes of the strain PN/90 were determined (see details under Materials and Methods) and compared to the genes of other influenza B virus isolates (Tables 1 and 2). Considering the regions analyzed (which did not include the sequences corresponding to the primers used for gene cloning, see legends to Tables 1 and 2), the nucleotide and predicted amino acid sequences of the NP, PB1, and PB2 genes contained neither insertions nor deletions as compared to the sequences previously determined for other B virus strains. However, the PN/90 PA gene lacks, as compared to the PA genes of strains AA/66 and SN/79, three consecutive noncoding nucleotides at the 5′ end of the genomic RNA. The PA proteins of strains PN/90 and AA/66 are 726 amino acids in length, whereas the SN/79 PA protein is one amino acid shorter.

TABLE 2

Number of Nucleotide (Lower Left Half) and Amino Acid (Right Upper Panel) Differences between Pairs of P Protein Genes

	PA		
	AA/66	SN/79	PN/90
AA/66	—	24	25
SN/79	82 (71)	—	8
PN/90	101 (91)	53 (44)	—
	PB1		
	LE/40	AA/66	PN/90
LE/40	—	12	14
AA/66	110 (107)	—	9
PN/90	152 (150)	78 (77)	—
	PB2		
	AA/66		PN/90
AA/66	—		7
PN/90	71 (70)		—

Note. The figures included in parentheses indicate the number of nucleotide substitutions in the coding regions. The sequences analyzed were: 2278 nt for the PA gene (from position 10 to 2287), 2266 nt for the PB1 gene (from position 30 to 2295), and 2366 nt for the PB2 gene (from position 10 to 2375). The nucleotide positions are given according to the sequences reported for the AA/66 strain. For comparisons of the PA genes, each of the nucleotide and amino acid deletions was considered as one nucleotide or amino acid difference. The EMBL/GenBank accession numbers for the genes analyzed were as follows: PB1 gene, M14880 (LE/40), M20479 (AA/66), AF005736 (PN/90); PB2 gene, M20168 (AA/66), AF005737 (PN/90); PA gene, M20478 (AA/66), M16711 (SN/79), and AF005738 (PN/90).

The nucleotide sequence of the PN/90 NP gene was compared to NP sequences previously reported (LE/40, AA/66, and SN/79) as well as to others (AA/86, YM/88, and TX/88), not yet published (P. A. Rota, personal communication), but available from the EMBL-GenBank. As can be seen in Table 1, the NP gene of PN/90 is closely related to that of the recent isolate YM/88, and it is also related, although to a lesser extent, to the NP gene of strains SN/79 and TX/88. Interestingly, the strain PN/90 shares a closer relationship with the old isolate SN/79 than with the strain AA/86, which was isolated only 4 years before than PN/90. This lack of correlation between date of isolation and the number of nucleotide and amino acid differences suggests the existence of cocirculating NP gene lineages. In fact, this appears to be the case, since analysis of the evolutionary relationships of the NP genes indicated in Table 1 reveals that the AA/86 strain is in a lineage different from that including the other recent virus strains (TX/88, YM/88, and PN/90) (Fig. 3).

Comparisons of the coding regions of the HA1 domain of HA genes of influenza B strains have revealed that two distinct HA evolutionary lineages (one including strains AA/86 and TX/88, and the other one including isolates YM/88 and PN/90) have been cocirculating in recent years (Kanegae *et al.,* 1990; Rota *et al.,* 1990, 1992; Kinnunen *et al.,* 1992). Thus, it was of interest to compare the phylogenetic trees for the HA1 and NP genes. In Fig.

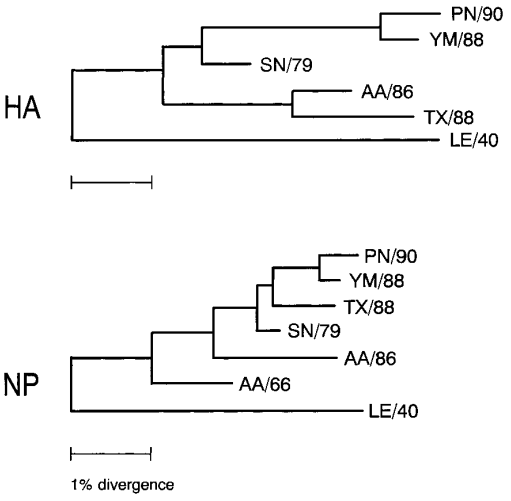

FIG. 3. Evolutionary trees for the NP and HA1 genes of influenza B viruses. For constructing the trees, the differences in nucleotide sequences between pairs of strains were calculated and these figures were analyzed by the neighbor-joining method (details are given under Materials and Methods). Horizontal branch lengths are drawn to scale (the scale bar represents, for both trees, 0.01 nucleotide substitutions per site), but vertical separation is for clarity only. The EMBL/GenBank accession numbers for the HA1 genes analyzed were as follows: J02093 (LE/40), X00897 (SN/79), M21874 (AA/86), M58419 (YM/88), M58425 (TX/88), and M65171 (PN/90).

3, it is shown the phylogenetic relationships of the HA1 nucleotide sequences for strains for which both the HA and NP gene sequences are known (all strain listed in Table 1, except AA/66). The branching pattern of the HA and NP trees is the same except for the position of the TX/88 strain. The HA1 gene of this strain is grouped together with that of the isolate AA/86, whereas the TX/88 NP gene is related to the genes of strains SN/79, YM/88, and PN/90 strains. This feature suggest that reassortment of the HA and/or NP genes has occurred among recent influenza B virus strains. It should be mentioned that Xu *et al.* (1993) suggested, by comparison of the endonuclease restriction patterns of PCR-amplified products corresponding to segment 5 and 7, that reassortment of the M and NP genes has also occurred among recent B virus isolates. However, no evidence of reassortment was found upon comparisons of the HA and NS gene sequences of a number of influenza B virus strains (Yamashita *et al.,* 1988).

Comparisons of the P genes of strains PN/90 and AA/66, the only two influenza B virus isolates for which the sequences of the three P genes have been determined, indicate that the percentage of nucleotide differences is similar for the three P genes (3.4, 3, and 4.1% for the PB1, PB2, and PA genes, respectively). However, at the amino acid level, the PB1 and PB2 proteins are more highly conserved (differing in 1.2 and 0.9% of the positions, respectively) than the PA proteins which differ in 4.8% of the amino acid residues. Therefore, these comparisons indicate there is a higher proportion of coding changes in the PA gene than in the PB1 and PB2 genes.

Summary

We have demonstrated here that the four influenza B virus core proteins are the minimum set of viral polypeptides required for expression of the viral genome, and it has also been shown that type B polymerase can recognize the influenza A virus promoter *in vivo.* Based on this latter fact, we speculate that it should be possible to generate influenza B viruses (as previously reported for influenza A virus by Muster *et al.,* 1991), containing viral genes flanked by type A cis-acting signals, which may have properties desirable for a virus vaccine.

Natural stable reassortant viruses containing influenza A and B virus RNA segments have never been described (Mikheeva and Ghendon, 1982; Kaverin *et al.,* 1983). The absence of reassortment between type A and B viruses is a complex phenomenon, which most likely involves type-specific interactions between several viral proteins. From the experiments described here, it is clear that one of these type-specific protein interactions involves the RNP protein components. We have shown that in order to obtain a functional polymerase, the four core proteins should be derived from the same virus type, and therefore it is concluded that it would not be possible to isolate a reassortant virus encoding a mixture of the type A and type B core proteins.

The results obtained from sequence analyses of the cloned genes indicate the existence of cocirculating NP gene lineages, and that genetic reassortment of the HA and/or NP genes had occurred among recent influenza B virus isolates.

Recently, it has been possible to rescue synthetic genes into infectious influenza B virus (Barclay and Palese, 1995) and to reconstitute influenza B virus RNPs *in vitro* (Lee and Seong, 1996). It is considered that the artificial expression system described here, in combination with the other two recent developments, should allow a detailed understanding of the RNA transcription processes of influenza B viruses.

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